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Staphylococcus aureus ClpC Is Required for Stress Resistance, Aconitase Activity, Growth Recovery, and Death

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The ability of *Staphylococcus aureus* to adapt to various conditions of stress is the result of a complex regulatory response. Previously, it has been demonstrated that Clp homologues are important for a variety of stress conditions, and our laboratory has shown that a *clpC* homologue was highly expressed in the *S. aureus* strain DSM20231 during biofilm formation relative to expression in planktonic cells. Persistence and long-term survival are a hallmark of biofilm-associated staphylococcal infections, as cure frequently fails even in the presence of bactericidal antimicrobials. To determine the role of *clpC* in this context, we performed metabolic, gene expression, and long-term growth and survival analyses of DSM20231 as well as an isogenic *clpC* allelic-replacement mutant, a *sigB* mutant, and a *clpC sigB* double mutant. As expected, the *clpC* mutant showed increased sensitivity to oxidative and heat stresses. Unanticipated, however, was the reduced expression of the tricarboxylic acid (TCA) cycle gene *citB* (encoding aconitase), resulting in the loss of aconitase activity and preventing the catabolization of acetate during the stationary phase. *clpC* inactivation abolished post-stationary-phase recovery but also resulted in significantly enhanced stationary-phase survival compared to that of the wild-type strain. These data demonstrate the critical role of the ClpC ATPase in regulating the TCA cycle and implicate ClpC as being important for recovery from the stationary phase and also for entering the death phase. Understanding the stationary- and post-stationary-phase recovery in *S. aureus* may have important clinical implications, as little is known about the mechanisms of long-term persistence of chronic *S. aureus* infections associated with formation of biofilms.

Staphylococcus aureus is a major human pathogen (33) that is unabated as a significant cause of morbidity and mortality in community-acquired and nosocomial infections. One reason contributing to its endemic importance is thought to be the organism's marked ability to survive under adverse environmental conditions (6). Another reason for the occurrence of the ubiquitous infections caused by *S. aureus* is its ability to adhere to inert surfaces of medical implantable devices (31) as well as its marked capability to colonize biologic substrates such as heart valves (20). On these inert or physiological surfaces, *S. aureus* may proliferate as a structured community of bacterial cells enclosed in a self-produced polymeric matrix termed biofilm.

In a previous work, using a micro-representational-difference approach, we have identified genes which are overexpressed in a *S. aureus* DSM20231 biofilm population compared to a planktonic population (3). Of the obtained difference

product pools, five genes were further characterized. One of these identified genes was a *clpC* homologue encoding the ATPase ClpC, a class III group heat shock protein (10). Clp (caseinolytic protease) (24) protein complexes play a crucial role in energy-dependent proteolysis, a common mechanism in prokaryotic and eukaryotic cells for intracellular homeostasis and regulation, particularly under stress conditions (17, 48). The Clp complex is composed of a proteolytic subunit, ClpP, which associates with a Clp ATPase. The latter comprise a protein family designated HSP100 proteins. While Clp ATPases alone have a substrate-specific chaperone function (i.e., refolding and reactivation of proteins), the Clp ATPase-ClpP complex confers protease activity (47). In *S. aureus*, several genes encoding Clp ATPase homologues have been identified: *clpC* (SA 0483 according to the *S. aureus* N315 genome designation [30]), *clpB* (SA 0835), *clpY* (SA 1097), *clpX* (SA 1498), and *clpL* (SA 2336). In addition, two genes encoding Clp proteases are present: *clpP* (SA 0723) and *clpQ* (SA 1096).

While little is known about the role of ClpCP in *S. aureus*, this complex was shown to play crucial roles in targeting MecA and ComK in *Bacillus subtilis* and thus controlling growth at high temperature, cell division, sporulation, competence for genetic transformation, and degradative-enzyme synthesis (28,

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TABLE 1. Bacterial strains and primers

Strain or primer	Genotype, relevant characteristics, or nucleotide sequence	Reference
Strains		
<i>S. aureus</i>		
DSM20231	Wild-type standard laboratory strain (DSMZ)	41
SH1000	8325-4 <i>rsbU</i> ⁺	21
PBM001	DSM20231 <i>clpC::ermB</i>	This study
HCH001	SH1000 <i>clpC::ermB</i>	This study
MGM001	PBM001(pCX19clpC)	This study
GP266	RN4220 <i>rsbU</i> ⁺ <i>sigB1</i> (Am) Tc ^r	4
MB290	DSM20231 <i>sigB1</i> (Am) Tc ^r	This study
MB288	PBM001 <i>sigB1</i> (Am) Erm ^r Tc ^r	This study
SA113	Restriction-negative mutant of <i>S. aureus</i> 8325-4	23
<i>S. carnosus</i> TM300	Invasion-negative control strain	40
Primers		
Cloning		
<i>MclpC</i> Forw	5'-ATATGGATCCTGTAGAATGATAACTATGGAAGAGG-3'	
<i>MclpC</i> 2Rev	5'-ATATGGATCCCAAATTCACCACGATATTTAGTACC-3'	
<i>clpC</i> 2	5'-ATATGGTACCTGGACTGTTTTATATTATGCTTGC-3'	
<i>ermB</i> For	5'-ATGAACAAAAATATAAAATATTCTCAAAAC-3'	
<i>ermB</i> Rev	5'-TTATTTCTCCTCCCGTTAAATAATAGATAAC-3'	
<i>KclpC</i> 1A	5'-ATATGGATCCAGTAGGAGGTCATTATTTATGTTATT-3'	
<i>KclpC</i> 2Sma	5'-ATATCCCGGGTGGACTGTTTTATAATTATGCTTGC-3'	
Real-time RT-PCR		
<i>acsA</i> f1	5'-AAGATATGCAACGGTTATCTAATAAAGCA-3'	
<i>acsA</i> r1	5'-AGGTGTACGCGACATAAAATATAAATACTCT-3'	
<i>asp23</i> f1	5'-CAAGAACAAAAATCAAGAGCCTCAAT-3'	
<i>asp23</i> r1	5'-CTTCACGTGCAAGCGATACCA-3'	
<i>citB</i> f1	5'-CATTACCACAAGGCGCAACA-3'	
<i>citB</i> r1	5'-GAACTCCACAAATTTACCAACAACA-3'	
<i>clpB</i> f1	5'-AGTAGCAGTTAGTGAGCCTGATG-3'	
<i>clpB</i> r1	5'-TCTATCTTGAATACGCACACCATG-3'	
<i>clpC</i> f1	5'-GAAGAAGCAATTTCGTTTAAATCATTCA-3'	
<i>clpC</i> r1	5'-CTTTCTAATACTTTTGCAGCAATTCCTT-3'	
<i>clpP</i> f1	5'-TGACAACGTAGCAAATTCATCGTAT-3'	
<i>clpP</i> r1	5'-CACTTCCACCTGGTGAATTAATGTAT-3'	
<i>gyrB</i> f1	5'-GACTGATGCCGATGTGGA-3'	
<i>gyrB</i> r1	5'-AACGGTGGCTGTGCAATA-3'	

34, 45). Furthermore, it is a general stress protein required for in vivo survival of *Listeria monocytogenes*, being involved in early bacterial escape from the phagosomes of macrophages (37, 38).

The regulation and biological function of *S. aureus* Clp ATPase homologues have been studied in different genetic backgrounds (7, 11, 15); however, in some of these studies a chemically mutagenized strain or an *rsbU* mutant lacking the positive regulator of σ^B activity was used. Additionally, many of the commonly utilized laboratory strains fail to form a biofilm (9), an important mechanism for causing disease. These observations led us to examine the role of ClpC in the life cycle of *S. aureus* without known underlying gene defects or alterations capable of biofilm formation (3).

(This work was performed as partial fulfillment of the requirements for the Ph.D. degree at the University of Saarland, Germany, for I. Chatterjee.)

MATERIALS AND METHODS

Bacterial strains and growth conditions. The parental strain used in this study was *Staphylococcus aureus* DSM20231 (ATCC 12208) (from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH [DSMZ], Braun-

schweig, Germany), which is a standard laboratory type strain (41). For starvation survival studies, *S. aureus* strains were grown in brain heart infusion (BHI) (Oxoid) medium or on Mueller-Hinton agar (containing 1.5% agar). All bacterial cultures were inoculated from an overnight culture and diluted to an optical density at 600 nm (OD_{600}) of 0.1 into BHI medium incubated at 37°C. For generation of aerobic growth condition, Erlenmeyer flasks (1 liter) were incubated at a flask-to-medium volume ratio of 10:1 and shaken at 230 rpm. Aliquots were removed at the indicated time points, and bacterial growth or CFU were determined. Bacterial growth was assessed by measuring the optical density at 600 nm. For stress tolerance studies, *S. aureus* strains were grown in tryptic soy broth (TSB) (Oxoid) under aerobic growth conditions or on tryptic soy broth containing 1.5% agar (TSA) incubated at 37°C. All strains used are summarized in Table 1.

DNA manipulations. DNA manipulations, DNA sequencing, PCRs, and plasmid isolation were performed using standard procedures (39) or according to manufacturers' instructions.

Construction of the *S. aureus* *clpC* mutant (PBM001). A 988-bp fragment (nucleotides -215 to +773 relative to the *clpC* start codon) was amplified from chromosomal DNA of *S. aureus* DSM20231 by PCR with primers (*MclpC*Forw and *MclpC*Rev [Table 1]) that contained restriction sites for BamHI and was cloned into pCRII (Invitrogen) to generate the plasmid pClpC. The 1,467-bp *ermB* cassette of pEC4 (5) was amplified using primers *ermB*For and *ermB*Rev (Table 1) and was inserted into the blunt-ended ClaI fragment contained within the *clpC* fragment of pClpC to yield pClpCII. The *clpC-ermB-clpC* fragment was isolated from pClpCII as a 2,455-bp BamHI-BamHI fragment and cloned into the temperature-sensitive shuttle vector pBT2 (5) to generate the plasmid

pBelpC. The plasmid pBelpC was propagated in strain SA113 (23) and then transferred to *S. aureus* DSM20231 by protoplast transformation (18). This was followed by integration of the *ermB* gene into the *S. aureus* chromosome and selection by a temperature shift (36). For verification, the PCR product of the *clpC* mutant was analyzed with primers *MclpC*Forw and *clpC2* (Table 1) and was found to be 1.47 kb larger than the product of the wild-type (WT) strain *S. aureus* DSM20231. Pulsed-field gel electrophoresis showed similar bands for both the WT and PBM001, and no deviation in the band pattern could be observed due to insertion of the *ermB* cassette. Finally, all ID 32 Staph-System tests gave reaction results that were identical for both the strains.

Construction of the *S. aureus sigB* mutant (MB290) and the *S. aureus clpC sigB* mutant (MB288). Strains MB290 and MB288 were obtained by phage 80 α -mediated transduction of the *tet*(L)-tagged *sigB1*(Am) mutation of GP266 (4) into DSM20231 and PBM001, respectively, with selection for tetracycline resistance.

Complementation of the *S. aureus clpC* mutant (MGM001). To create a *clpC* expression vector for complementation, the *clpC* gene from *S. aureus* DSM20231 was amplified by PCR. Additional restriction sites with primers *KclpC1A* (BamHI) and *KclpC2Sma* (SmaI) (Table 1) were introduced. The vector part was derived from the expression vector pCX19 (22) by excision of the lipase gene with BamHI and SmaI. The restricted PCR product was ligated into the restricted pCX19 vector. The restriction-negative strain *S. carnosus* TM300 (40) was transformed with this ligation product (pCX19*clpC*) by protoplast transformation (18). After analysis of the expression vector pCX19*clpC* by sequencing, the *clpC* mutant (PBM001) was transformed with pCX19*clpC* by electroporation, resulting in the transformant MGM001. Transformants were verified by plasmid preparation and subsequent restriction.

Real-time reverse transcription-PCR (RT-PCR). For RNA isolation from culture, *S. aureus* was grown in BHI medium to the desired growth phase (2, 6, 16, 48, 72, and 96 h). Bacteria were mechanically disrupted (Fast Prep FP120 instrument; Qiogene, Heidelberg, Germany) and RNA isolated (RNeasy mini-kit; QIAGEN, Hilden, Germany). After treatment with RNase-free DNase I (QIAGEN), total RNA samples were amplified in an ABI PRISM 7000 Sequence Detection System, using SYBR Green PCR master mix (Applied Biosystems, Weiterstadt, Germany) and *gyrB* primers (Table 1) to check for absence of gDNA. Previously transcribed cDNA served as a positive control. RNA was then reverse transcribed (High Capacity cDNA Archive Kit; Applied Biosystems). cDNA was used for real-time amplification with specific primers (Table 1) and 100 ng of cDNA per reaction. The levels of mRNA expression of the different genes were normalized against expression of the internal control *gyrB*, which is constitutively expressed (49). The amounts of different transcripts were expressed as the *n*-fold difference relative to the control gene ($2^{-\Delta CT}$, where ΔCT represents the difference in threshold cycle between the target and control genes).

Determination of stationary-phase survival. Single colonies of *S. aureus* strains were inoculated into 1-liter flasks containing 100 ml of BHI medium, grown at 37°C, and aerated by shaking at 230 rpm for up to 1 week. Aliquots (200 μ l) were taken at different time intervals (23, 42, 48, 65, 70, 96, 120, 144, and 168 h), and the CFU per milliliter were determined on Mueller-Hinton agar plates.

Determination of glucose and acetate in culture supernatants. Assays were performed as described previously (42). Briefly, aliquots of bacteria (2 ml) were centrifuged for 5 min at $21,000 \times g$ at 4°C at the indicated time points. The culture supernatants were removed and adjusted to pH 8 with KOH. Glucose and acetate concentrations were determined with commercial kits (R-Biopharm AG, Darmstadt, Germany).

Aconitase activity assay. The aconitase activity assay was performed as previously described, with slight modifications (26). Briefly, aliquots (4 ml) were harvested at the different time points (24, 48, 72, and 96 h) and resuspended in 1.5 ml of aconitase lysis buffer (ACN) containing 90 mM Tris (pH 8.0) and 100 μ M fluorocitrate. Bacteria were mechanically disrupted (Fast Prep) as stated above. The lysate was centrifuged for 5 min at $21,000 \times g$ at 4°C. Aconitase activity in the resulting cell-free lysate was assayed by the method described previously (26). One unit of aconitase activity is defined as the amount of enzyme necessary to yield a $\Delta A_{240} \text{ min}^{-1}$ of 0.0033 (1). Protein concentrations were determined by the method of Lowry et al. (32).

RESULTS

***clpC* inactivation impairs post-stationary-phase recovery under aerobic conditions.** Long-term survival of the *S. aureus* WT strain (DSM20231) as well as of the *S. aureus clpC* mutant strain (PBM001) was determined for up to 7 days under aer-

obic conditions. The two strains grew at similar rates during the exponential growth phase. Between 24 h and 48 h (stationary phase), cell density (OD₆₀₀) decreased in all strains. At 72 h, cell densities of the WT again increased until 96 h, indicating a recovery in this post-stationary phase, and remained constant for up to 7 days, while PBM001 was unable to grow following the stationary phase (Fig. 1A). Additionally, we assessed stationary-phase survival of the *clpC* mutant and the WT. Single colonies were inoculated into BHI medium and grown aerobically for 1 week, and viable counts were determined daily. CFU values for the WT coincided with growth curve results, clearly demonstrating a decrease in viable counts during the stationary phase (24 h to 48 h), followed by a significant increase during the recovery in the post-stationary phase (48 h to 72 h) and finally entering the death phase. In contrast, the *S. aureus clpC* mutant lacked recovery during the post-stationary growth phase but survived to a significantly greater extent during prolonged growth (>72 h) (Fig. 1B). This observation was further extended by introducing the *clpC* mutation in a different *S. aureus* background by phage 85-mediated transduction of the *ermB*-tagged *clpC* insertion mutation of PBM001 into *S. aureus* SH1000 (an *rsbU*⁺ derivative of 8325-4 [21]) and selecting for erythromycin resistance. This SH1000 *clpC* mutant (designated HCH001) also survived during extended times of growth (>72 h) (data not shown). The abrogated post-stationary-phase recovery in PBM001 was partially restored in the complemented mutant, MGM001, with an increase in cell density (Fig. 1A) and viable counts (Fig. 1B) at 72 h, attainment of intermediate cell density levels during the post-stationary-phase recovery, and then entering the death phase. The pH changes were almost identical for all strains (pH 5.9 to 6.0 at 6 h to 8 h [effect of glycolysis], followed by catabolism of the L-amino acids in the medium leading to an increase in the pH to 8.5 to 9.0 by 168 h). The alternative sigma factor σ^B has been recognized as a general stress-responsive sigma factor (15, 25). Therefore, we compared the σ^B -negative phenotype to a ClpC-negative phenotype by constructing a *sigB* mutant and a *clpC sigB* double mutant (MB290 and MB288, respectively). The growth pattern of MB290 was similar to that of the wild-type strain, while the growth pattern of MB288 was identical to that of the *clpC* mutant strain (Fig. 1).

These data clearly demonstrate that the *S. aureus clpC* mutant and the *clpC sigB* double mutant both failed to grow after stationary phase and failed to enter into the death phase. As Clp analogues have been demonstrated to be of importance in resistance to oxidative stress (11) as well as to thermotolerance and osmotic stress (12), and in order to confirm the functionality of the *clp* gene system in our WT strain and the mutants, we compared the stress sensitivities under various conditions.

***clpC* inactivation causes increased sensitivity to oxidative stress.** A disk diffusion assay with two different concentrations of hydrogen peroxide (15% and 30%) was performed as previously described (11). After inoculation of TSA plates with stationary-grown cells (24 h, 37°C, 230 rpm), H₂O₂ impregnated disks (10 μ l) were placed and the plates were incubated (37°C, 18 h). Both *clpC* (PBM001) and *sigB* (MB290) mutant cells displayed increased sensitivity to both hydrogen peroxide concentrations tested, while the WT was more resistant to oxidative stress (Fig. 2A and B). The *clpC sigB* double mutant (MB288) did not display any additional increase in inhibition

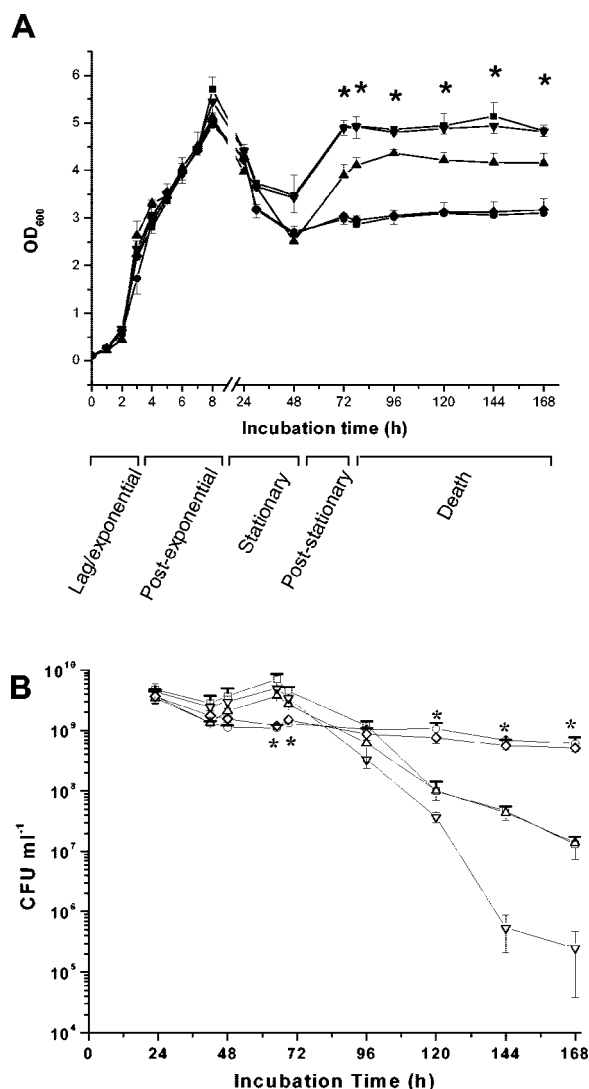


FIG. 1. A. Growth analysis of the WT and the *clpC* and *sigB* mutants. Growth curves (OD₆₀₀) of WT *S. aureus* DSM20231 (■), the *clpC* mutant (PBM001; ●), the *sigB* mutant (MB290; ▼), and the *clpC sigB* double mutant (MB288; ◆) were determined in BHI medium. For analysis of the *clpC*-complemented strain in PBM001 (MGM001; ▲), medium was supplemented with xylose (1%). Data are means \pm standard errors of the means of values obtained in three independent experiments *, $P < 0.001$ compared to WT (t test). B. Inactivation of *clpC* enhances stationary-phase survival. Single colonies of WT *S. aureus* DSM20231 (□), the *clpC* mutant (PBM001; ○), the *sigB* mutant (MB290; ▽), and the *clpC sigB* double mutant (MB288; ◇) were inoculated into BHI medium, grown at 37°C, and aerated by being shaken at 230 rpm for up to 1 week. At different intervals, aliquots were removed and CFU per milliliter were determined in triplicate. For analysis of the *clpC*-complemented strain in PBM001 (MGM001; △), medium was supplemented with xylose (1%). Data are means \pm standard deviations of values obtained in two independent experiments *, $P < 0.05$ compared to WT (t test).

zone size compared to PBM001. Similar results were obtained when the oxidative stress responses of SH1000 and its *clpC* mutant HCH001 were examined (data not shown).

In addition to the plate assay, growth of the WT and PBM001 was also determined in liquid BHI medium with or

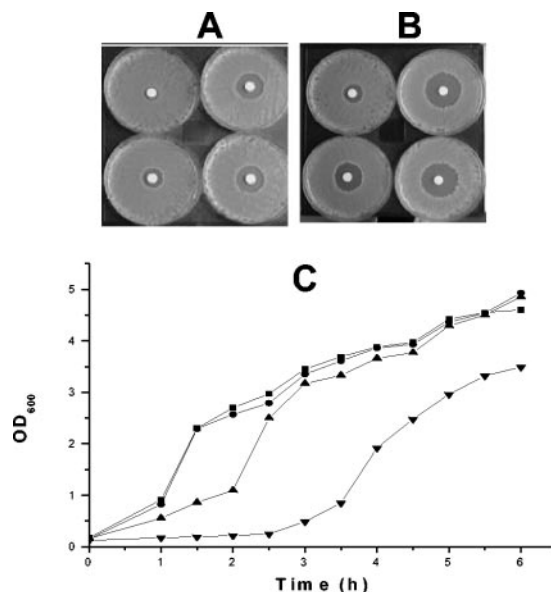


FIG. 2. Sensitivity of *clpC* or *sigB* mutants to oxidative stress. (A and B) Cells of the WT (upper left plates), PBM001 (*clpC* mutant; upper right plates), MB290 (*sigB* mutant; lower left plates), and MB288 (*clpC sigB* double mutant; lower right plates) grown for 24 h (in TSB) were plated on TSA. A disk containing 10 µl of hydrogen peroxide at a concentration of either 15% (A), or 30% (B) was placed, and then plates were incubated (37°C, 18 h). (C) Fifty-milliliter cultures of the wild type and the *clpC* mutant were grown to an OD₆₀₀ of 0.1. The cultures were split, 7.5 mM of hydrogen peroxide was added to one half, and then both halves were incubated (37°C). Shown are OD₆₀₀ values obtained at the indicated time points (■, WT without H₂O₂; ▲, WT with H₂O₂; ●, PBM001 without H₂O₂; ▼, PBM001 with H₂O₂). Shown are representative results of at least two independent experiments.

without hydrogen peroxide supplementation (Fig. 2C). In agreement with the results obtained in the disk diffusion assay, PBM001 displayed increased susceptibility to hydrogen peroxide, with a clear growth retardation by approximately 2.5 h.

Heat shock reduces growth in the *clpC* mutant and enhances *clpC*, *clpB* and *clpP* transcript expression. Generally, *clp* homologues are responsive to heat shock (7, 11, 12, 19). It has previously been reported that *clpB*, *clpC*, *clpL*, and *clpP* genes are induced by heat shock in *S. aureus* COL (15), RN4220 (7), and 8325-4 (11). Thus, we compared gene expression of *clpC*, *clpP*, *clpB*, *clpX*, and *clpL* in WT *S. aureus* as well as in the mutants either grown at 37°C or exposed to 55°C for 15 min. Real-time quantitative PCR was used to examine the modulation of gene expression in *S. aureus*. The levels of mRNA expression of the different genes were normalized against the constitutively expressed internal control *gyrB* (49). A 15-fold up-regulation of the *clpC* transcript was noted 5 min after heat shock in the WT, followed by a rapid repression of the transcript by 30 min after heat shock (Fig. 3E). No detectable expression was found for the corresponding genes in the samples without heat shock (data not shown). Similarly to *clpC*, following heat shock, expression of *clpB* and *clpP* was up-regulated in the WT and the *clpC* mutant (Fig. 3E) as well as in the other mutant strains (MB290 and MB288 [not shown]) and then decreased. Similar to previously published data (11), *clpX* was not induced after heat shock at detectable levels in

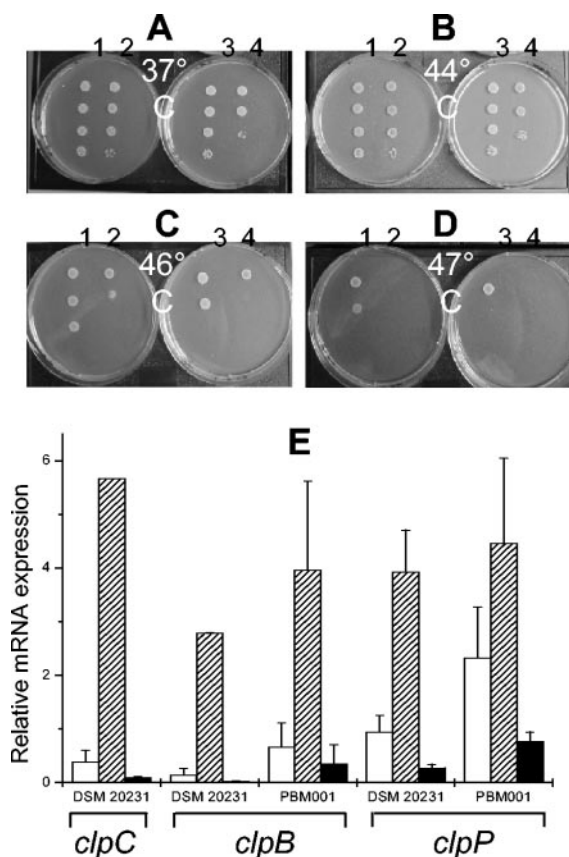


FIG. 3. Growth and *clp* gene expression after heat shock. (A to D) Cultures of the WT (1), PBM001 (2), MB290 (3), or MB288 (4) were grown to the exponential phase in TSB at 37°C. At an OD₆₀₀ of 0.25 to 0.30, cultures were diluted 10⁻¹, 10⁻², 10⁻³, and 10⁻⁴-fold, and 10 μ l of each dilution (top to bottom, respectively) was spotted on TSA plates. The plates were then incubated at 37°C (A), 44°C (B), 46°C (C), and 47°C (D) for 24 h. (E) Real-time RT-PCR quantification of *clp* gene expression after heat shock at 55°C for 15 min. *clpC*, *clpP*, and *clpB* mRNA concentrations in the WT (DSM 20231) and the *clpC* mutant (PBM001) after heat shock at 0 (\square), 5 (\square), and 30 (\blacksquare) min were determined as described in Materials and Methods. Shown are transcript quantities relative to the control (*gyrB*) transcript, expressed as fold increase. Data are means \pm standard deviations of triplicate determinations.

either strain (data not shown). In contrast to results obtained using *S. aureus* strain COL (15), we found that heat stress did not induce the expression of *clpL* (data not shown).

The increased expression of *clpC* following a heat shock suggested that the *clpC* mutant strain would be sensitive to heat stress. To test this hypothesis, we subjected the WT, *clpC* (PBM001), *sigB* (MB290), and *clpC sigB* (MB288) strains to elevated temperatures and examined the effects on growth (Fig. 3A to D). At 37°C, the WT, PBM001, and MB290 grew to similar extents; however, at elevated temperatures, the viability of the mutant strains was significantly reduced (approximately 100-fold reduction) relative to that of the WT strain (Fig. 3D).

ClpC is required for acetate catabolism and aconitase activity. Previously we have shown that *S. aureus* post-exponential-phase growth requires an intact tricarboxylic acid (TCA) cycle to facilitate the catabolism of secondary metabolites (e.g., acetate) (42, 43). Based on these observations and on the fact

that WT strain DSM20231 grew after a brief stationary phase (Fig. 1A), we hypothesized that the post-stationary-phase recovery was dependent upon the catabolism of secondary metabolites. To test this hypothesis, we (i) analyzed the levels of growth metabolites (glucose and acetate) of the TCA cycle in the supernatants of the *S. aureus* mutants as well as expression of *acsA*, encoding the acetate-utilizing enzyme acetyl coenzyme A (acetyl-CoA) synthetase; (ii) determined the gene expression levels of *asp23*, *citB*, and *clpC*; and (iii) performed an aconitase activity assay.

In accordance with published data (43), glucose was catabolized in the exponential phase of growth in all strains and was completely consumed by 4 h (<0.05 mM). The *clpC*-functional strains DSM20231, MGM001 (complemented mutant), and MB290 (*sigB* mutant) started to catabolize acetate after 48 h, and by 96 h, the acetate was depleted from the culture medium (Fig. 4A). Elevated levels of *acsA* expression in *clpC*-positive strains (WT and MB290) support this observation (Fig. 4B). In striking contrast, the two *clpC*-defective mutants PBM001 and MB288 failed to catabolize acetate (Fig. 4A and B), suggesting that *clpC* inactivation impaired TCA cycle function. Accordingly, we examined the mRNA abundances of *citB*, encoding the TCA cycle enzyme aconitase, and *clpC* in the WT, *clpC* mutant (Fig. 5A and B), and *sigB* mutant strains (not shown). Additionally, as a control we examined the expression of *asp23*, whose transcription is controlled exclusively by the level of free σ^B (14, 16). As predicted, the transcription of *citB* increased in the WT strain, coinciding with the decrease in acetate levels (Fig. 4A). Transcript levels of *citB* in PBM001 also increased at 48 h, albeit to a lesser extent, and remained stable at later time points. *asp23* expression in the WT and PBM001 and *clpC* expression in the WT showed a pattern similar to *citB* expression, with an increase after the stationary phase. In the *sigB* mutant, as anticipated, *asp23* expression was very low throughout the growth cycle (data not shown).

The *citB* gene expression data were extended by functional assays. Aconitase activity was determined throughout the growth cycle (Fig. 6) and was found to be maximal at 72 h in the WT. In contrast, the *clpC* mutants (PBM001 and MB288) showed greatly reduced activity throughout the culture period. Aconitase activity in the *sigB* mutant initially increased; however, this activity was not sustained after 48 h.

DISCUSSION

In this paper, a role of ClpC in post-stationary-phase growth recovery and death is described, and these effects could be related to the activity of aconitase, resulting in a functional TCA cycle. Furthermore, a σ^B -independent effect of ClpC on stress resistance could be demonstrated. To our knowledge, this is the first report implicating an *S. aureus* chaperone as being necessary for metabolism and growth following the stationary phase. An appraisal of these findings includes the following considerations.

Recovery from the stationary phase and cell death. During the exponential phase of growth, *S. aureus* preferentially catabolizes glucose via glycolysis to generate pyruvate (27, 43). Under aerobic growth conditions, pyruvate is catabolized to acetyl-CoA and subsequently to acetyl-phosphate (13, 43). Acetyl-phosphate is used for substrate-level phosphorylation,

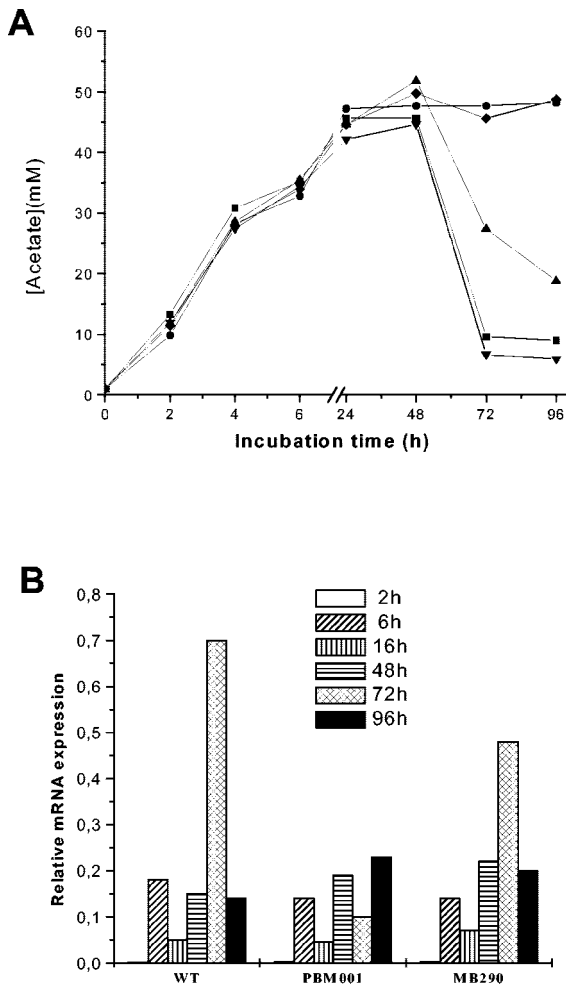


FIG. 4. A. Determination of acetate in the culture supernatant. After the indicated time intervals, supernatants of the WT (■), PBM001 (●), MGM001 (▲), MB290 (▼), and MB288 (◆), cultivated in BHI medium, were analyzed for acetate concentration as described in Materials and Methods. Shown are representative results of at least two independent experiments. B. Expression of acetyl-CoA synthetase (*acsA*) in the WT, PBM001, and MB290. Gene expression of *acsA* was determined in the WT, PBM001 (*clpC* mutant), and MB290 (*sigB* mutant) by real-time RT-PCR at different time intervals as described in the legend to Fig. 3.

generating ATP and the by-product acetate. Consistent with these observations, our data demonstrate a rapid depletion of glucose from the culture medium and a concomitant increase in the concentration of acetate. Derepression of the TCA cycle occurs upon depletion of the readily catabolizable carbon source(s) and/or glutamate and coincides with the depletion of acetate from the culture medium. It is thought that the depletion of rapidly catabolizable carbon sources or possibly multiple nutrient limitations as well as an acidic pH contributes to the decrease in the number of viable cells during the stationary phase (46). As expected, the cell densities and the viable counts of the WT and mutant strains decreased upon entry into stationary phase. Subsequently, *S. aureus* may display growth recovery (as also observed in *S. aureus* UAMS-1 [36a]). The prevention of growth recovery and the stationary-phase survival in the *clpC* mutant, however, can be related to the find-

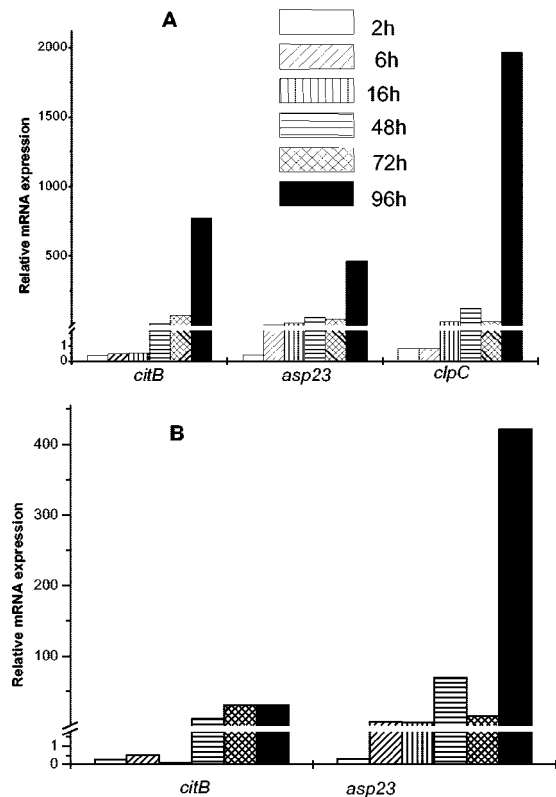


FIG. 5. Expression of *citB*, *asp23*, and *clpC*. Gene expression in the WT (A) and the *clpC* mutant PBM001 (B) was determined by real-time RT-PCR as described in the legend to Fig. 3. Expression of *clpC* in PBM001 was not determined.

ings of our previous study using a *S. aureus citB* mutant (42), which demonstrated enhanced stationary-phase survival similarly to that of the *clpC* mutant and the *clpC sigB* mutant in this study. This should be seen in the context of previous reports on stationary-phase survival in *B. subtilis*: the lack of expression of

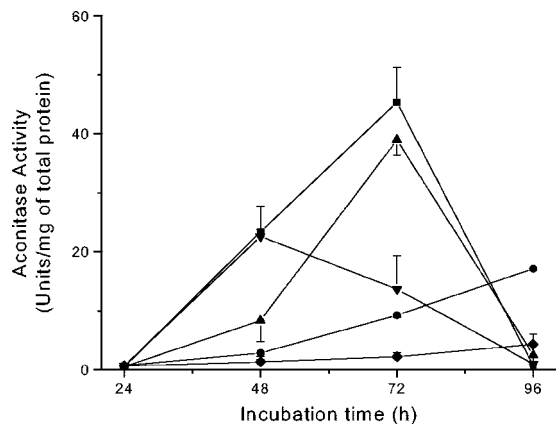


FIG. 6. Aconitase activity of the WT and *clpC* or *sigB* mutants. The WT (■), PBM001 (●), MGM001 (▲), MB290 (▼), and MB288 (◆) were grown as described in the legend to Fig. 1 for 24 h, 48 h, 72 h, and 96 h. Aconitase activities were determined as described in Materials and Methods in triplicate analysis. Results are means \pm standard errors from two independent experiments.

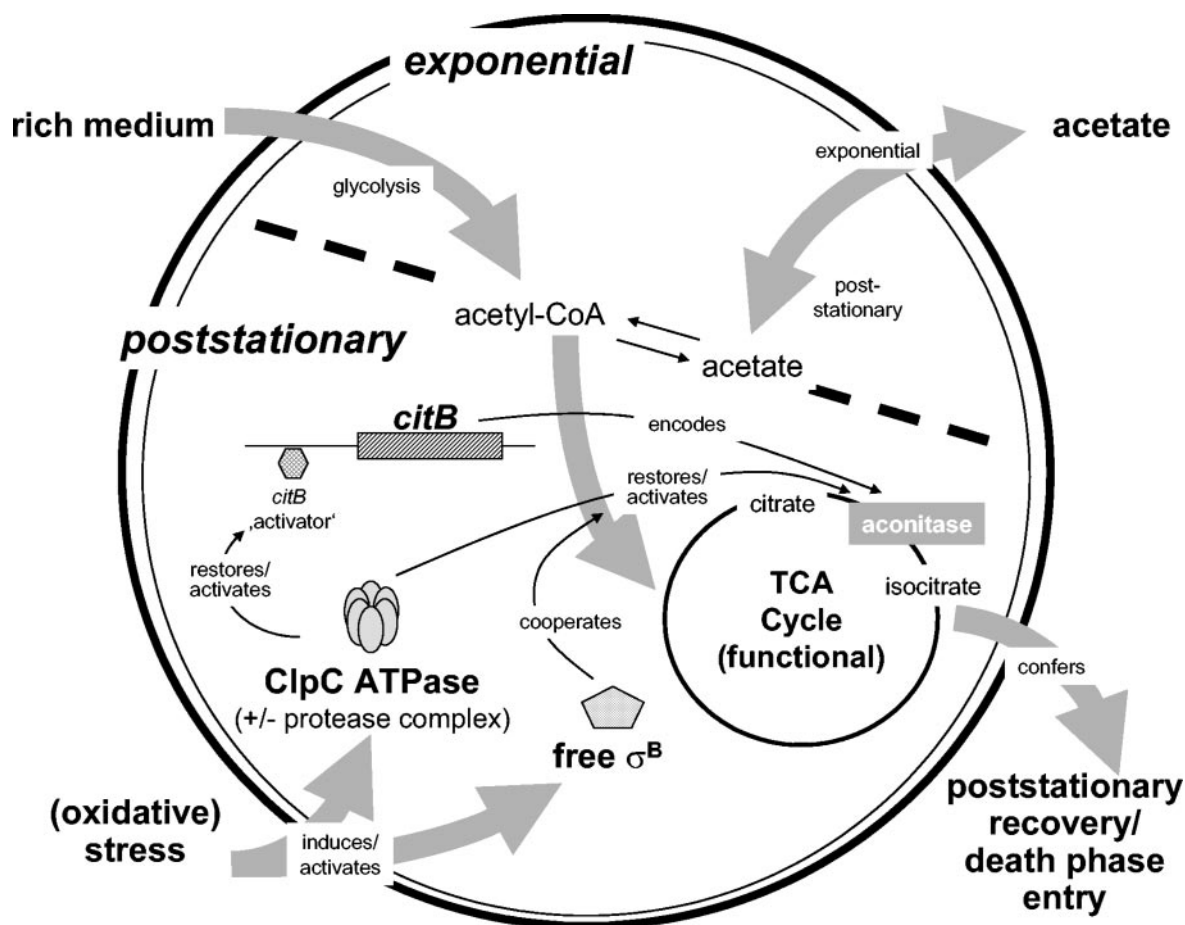


FIG. 7. Hypothetical model of the role of ClpC in oxidative metabolism of *S. aureus*. During exponential growth in rich medium, glucose is rapidly catabolized and finally accumulates as acetate in the medium. During late stationary phase (such as in biofilm populations), the bacterial cell yields free ATP primarily by oxidative metabolism provided by TCA cycle activity. The function of the key TCA cycle enzyme aconitase requires ClpC, since functional deletion of *clpC* results in a marked reduction in *citB* (aconitase) transcription along with loss of aconitase activity and persistent acetate accumulation. Under oxidative stress conditions, ClpC might be restoring/activating a yet-unknown activator(s) of *citB*, which in turn encodes active aconitase, and/or activating/restoring the activity of aconitase protein. This confers post-stationary-phase growth followed by entry into death phase. Free σ^B also seems to cooperate in activating/restoring the aconitase activity, through yet-unknown pathways.

citB (8) or *clpP* (35) in the respective *B. subtilis* mutants was shown to account for the loss of post-stationary-phase growth and/or survival. However, important species differences in metabolism or the differential role of *clpP* versus *clpC* might account for the apparent discrepancies with our findings for *S. aureus*. In a further extension of our earlier work (42), it therefore seems that TCA cycle inactivation due to a lack of ClpC completely prevents post-stationary-phase recovery in a complex medium and that the TCA cycle is essential for entry into the death phase.

Stress resistance. In the stationary phase, bacteria are exposed to a variety of environmental stress factors such as nutrient limitation and accumulation of toxic bacterial metabolic products, particularly reactive oxygen species (2). Clp protein complexes are important for reactivation and refolding damaged proteins under stress conditions (19). The importance of the *S. aureus* heat shock proteins ClpX and ClpP in stress tolerance and virulence has been demonstrated (11). Interestingly, in that report the absence of the ClpX ATPase conferred enhanced growth at either elevated temperatures or higher

puromycin concentrations. It was suggested that a Clp ATPase different from ClpX cooperates with ClpP in the degradation of stress-damaged proteins and that ClpX antagonizes this cooperation. Our data demonstrate that inactivation of ClpC enhances sensitivity to heat shock, suggesting that ClpC may be the protein cooperating with ClpP ATPase. This contention is supported by the observation that following a heat shock, expression of *clpP* and *clpC*, but not that of *clpX*, was enhanced.

In contrast to a previous report (15), we found that heat stress did not induce the expression of *clpL*. This apparent contradiction might be explained as follows. σ^B -dependent gene expression in response to heat shock is known to be transient, being highest shortly after the heat stress is applied (within 3 to 6 min) and decreasing thereafter to or below the uninduced level (>12 min) (14, 16). In our studies, the heat shock was applied for 15 min, a period that might have been too long to detect heat shock-mediated σ^B -dependent effects on *clpL* expression. Alternatively, culture media and growth conditions might have caused the lack of a heat shock effect on *clpL* (14; M. Bischoff, unpublished results).

Further, it was reported that *S. aureus* resistance to hydrogen peroxide was not dependent on ClpC (12). Those experiments were performed with exponential-phase SH1000 and 8325-4, and we could confirm the results using both SH1000 and DSM20231 (not shown). However, when using stationary-phase organisms, ClpC indeed was important for growth in presence of hydrogen peroxide, as shown in this paper. Thus, the growth phase appears to be crucial for the role of ClpC in stress resistance to hydrogen peroxide.

Role of *clpC* and σ^B in post-stationary-phase survival and recovery. The alternative sigma factor σ^B has been shown to be important for responding to environmental stimuli (e.g., pH or heat shock), growth phase-specific signals (e.g., stationary phase of growth), and ATP limitation conditions in *B. subtilis* (19) and in *S. aureus* (29). Therefore, in addition to the analysis of ClpC, we examined the role of σ^B in post-stationary-phase recovery. In fact, in contrast to data obtained for *clpC*, these results do not suggest a role of σ^B during these phases of growth. Furthermore, the impact on survival after oxidative and heat stresses was more pronounced in the *clpC* mutant than in the *sigB* mutant. Thus, we conclude that the effect of ClpC on late-stationary-phase growth and survival as well as on stress resistance is independent of cooperativity with σ^B .

Oxidative metabolism. In addition to its role in stress resistance in *S. aureus*, ClpC may have a more specific role in the oxygen-dependent bacterial metabolism at late stationary phase. Our finding that *citB* transcript levels were elevated in late stationary phase concurs with our previous reports on a functional aconitase and TCA cycle in *S. aureus* (42). Compared to that in the WT, however, *citB* expression during late stationary phase was significantly reduced in the *clpC* mutant. Moreover, while no difference was observed with respect to glucose catabolism, both the *clpC* and the *clpC sigB* mutants (but not the *sigB* mutant) failed to catabolize acetate during post-stationary-phase recovery. These findings concur with those from our recent study where we have shown that an *S. aureus* $\Delta citB$ mutant failed to grow postexponentially and to catabolize acetate in a rich medium, demonstrating that a fully functional TCA cycle is essential for acetate catabolism (27, 42). Our assumption, i.e., a functional defect of the TCA cycle activity in the *clpC* mutant, could subsequently be confirmed upon demonstration of a marked reduction of aconitase activity and restoration in the *clpC*-complemented mutant. Taken together, our observations clearly demonstrate the regulation of the activity of the TCA cycle enzyme aconitase by ClpC. They also indicate that aconitase activity, albeit not post-stationary-phase recovery is in part σ^B dependent, an observation in accordance with our previous data (43).

Possible role of ClpC in post-stationary-phase recovery. The mechanism by which ClpC exerts this profound effect on aconitase activity and the resulting changes in TCA metabolism have yet to be further defined. ClpC might be acting in several ways. It could be a chaperone for CitB (aconitase), which is a DNA binding protein and could be reversibly inactivated by oxidative stress (due to presence of the [4Fe-4S] cluster). ClpC might also be restoring/activating regulators of *citB* expression and activity (Fig. 7). The observed important reduction in transcription of the *citB* gene (Fig. 5) (25.3-fold decreased compared to wild type at 96 h) in the *clpC* mutant suggests that the absence of ClpC during the late stationary phase might

lead to energy limitation due to compromised TCA cycle activity. Overall, this might explain the demonstrated role of ClpC in the observed energy-requiring phenomenon of post-stationary-phase recovery followed by entry into the death phase.

Our findings may have important implications for the in vivo situation. Recently, we have shown that aconitase inactivation not only causes reduced cell densities postexponentially but also impairs the production of secreted *agr*-dependent virulence factors (42, 43). Moreover, impaired aconitase activity may suppress neutrophil migration normally elicited by the aconitase-dependent production of formylated peptides such as δ -toxin (44). Thus, the fine-tuning of ClpC activity, e.g., due to modulation of *clpC* expression in established biofilms (3), not only may be important for post-stationary-phase recovery in inanimate and in vivo environments but might also influence a number of *S. aureus* virulence mechanisms. Hence, our findings on ClpC modulation of oxidative metabolism extend our understanding of the overall physiology of this pathogen and can be related to other metabolic adaptive mechanisms resulting in "small-colony variant" or persister states.

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